

The role of protein kinase C in the induction of VCAM-1 expression on human umbilical vein endothelial cells

Theresa A. Deisher^{a,*}, Terri L. Haddix^b, Kevin F. Montgomery^b, Timothy H. Pohlman^b,
Kenneth Kaushansky^a, John M. Harlan^a

^a*Department of Medicine/Division of Hematology, Rm. 10, University of Washington Health Sciences, 1959 N.E. Pacific St., Seattle, WA 98195, USA*

^b*Department of Surgery, University of Washington, Seattle, WA 98195, USA*

Received 5 August 1993

The role of protein kinase C (PKC) in interleukin-1 β (Il-1 β), tumor necrosis factor- α (TNF- α), and lipopolysaccharide (LPS)-induced vascular cell adhesion molecule-1 (VCAM-1) expression on human umbilical vein endothelial cells (HUVEC) was studied. PKC inhibition or downregulation diminished VCAM-1 mRNA accumulation and protein expression. Interleukin-1 β , TNF- α , and LPS induce nuclear factor (NF)- κ B-like binding activity, which precedes VCAM-1 transcription. PKC inhibition did not prevent NF- κ B-like binding activity, indicating that this is PKC-independent, and NF- κ B-like binding activity is insufficient for transcription of VCAM-1.

Protein kinase C; Vascular cell adhesion molecule-1; Tumor necrosis factor- α ; Interleukin-1 β ; Lipopolysaccharide; Human umbilical vein endothelial cell

1. INTRODUCTION

Stimulation of human umbilical vein endothelial cells (HUVEC) by interleukin-1 β (Il-1 β) and tumor necrosis factor- α (TNF- α) induces a prothrombotic and proinflammatory phenotype. The proteins expressed by HUVEC following stimulation with these cytokines include tissue factor, plasminogen activator inhibitor-1, interleukin-8, interleukin-6, granulocyte-macrophage colony stimulating factor, and the adhesion molecules intercellular adhesion molecule-1 (ICAM-1), endothelial leukocyte adhesion molecule-1 (E-selectin), and vascular cell adhesion molecule-1 (VCAM-1) [1]. Although the pattern of Il-1- and TNF-induced surface protein expression has been well characterized, the intracellular signal transduction pathways utilized by Il-1 and TNF are unknown.

A potential role for PKC in mediating activation of HUVEC by Il-1 β and TNF- α is suggested by the fact that activators of PKC such as phorbol esters and mezerein are also reported to induce expression of ICAM-1 and E-selectin in HUVEC [2–4]. Additionally, inhibition of PKC by the isoquinoline-sulfonamide derivative H7 reduces leukocyte adherence to LPS-, TNF-, or Il-1-stimulated HUVEC [5], and inhibition or downregulation of PKC inhibits TNF- α , Il-1-, and LPS-induced endothelial cell expression of ICAM-1 [6]. We now re-

port that inhibition of PKC or downregulation of PKC by pretreatment with PMA significantly reduced the ability of TNF- α , Il-1 β , or LPS to induce the surface expression of VCAM-1 in HUVEC. Likewise, inhibition of PKC or pretreatment with PMA reduced TNF- α -induced accumulation of VCAM-1 mRNA. In contrast, induction of the surface expression of E-selectin by TNF- α , Il-1 β and LPS was not diminished by inhibitors of PKC.

2. MATERIALS AND METHODS

2.1. Culture of endothelial cells and ELISA

Human umbilical vein endothelial cells were obtained and cultured as previously described [7]. Cytokines, LPS, or phorbol esters were added to confluent monolayers of HUVEC 30 min after the addition of staurosporine (Calbiochem Corp., San Diego, CA), calphostin C (Kamiya Biomedical Co., Thousand Oaks, CA), or Ro31-7549 (Roche Products Ltd., Welwyn Garden City, UK) without washing. For downregulation studies, cytokines or LPS were added to the wells 24 h after the addition of PMA (100 ng/ml) (Sigma Chemical Co., St. Louis, MO), without washing. Adhesion molecule expression was measured by ELISA using the anti-E-selectin monoclonal antibody (mAb) BB11 [8] (a gift of R. Lobb and C. Benjamin, Biogen, Inc., Boston, MA) or the anti-VCAM-1 mAb 4B9 [7].

2.2. Total protein synthesis

Total cellular protein synthesis was determined by precipitation of [³⁵S]methionine-labeled proteins with trichloroacetic acid (10%). HUVEC were pre-incubated with 10 μ Ci/ml [³⁵S]methionine (DuPont Co., Wilmington, DE), for at least one hour prior to the addition of the protein kinase C inhibitors, and then TNF- α (10 ng/ml) was added for 4 h. The HUVEC were lysed and [³⁵S]methionine incorporation was determined by precipitation with trichloroacetic acid, followed by solubilization with 1 N NaOH and β -scintillation counting.

*Corresponding author. Fax: (1) (206) 685-3062.

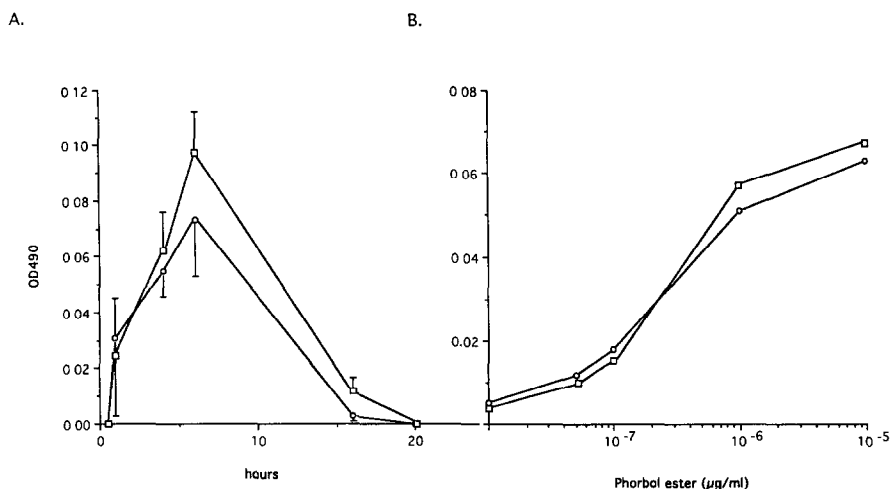


Fig. 1. Phorbol ester-induced surface expression of VCAM-1. Human umbilical vein endothelial cells were treated with 10 ng/ml to 10 µg/ml PMA or PDBU for up to 24 h. Surface expression of VCAM-1 was measured by ELISA using the anti-VCAM-1 MoAb 4B9. (A) Time-course for induction of surface expression of VCAM-1 by PMA (circles) or PDBU (squares) (1 µg/ml) (means ± S.E.M. of 5 experiments). (B) Concentration-dependent induction of VCAM-1 after a 5.5 h incubation with PMA (circles) or PDBU (squares) at 37°C (mean of 2 experiments).

2.3. Northern analysis

For Northern analysis, HUVEC were plated on gelatin-coated dishes and incubated with $\text{IL-1}\beta$, $\text{TNF-}\alpha$, LPS or PMA for 4 h. Total cellular RNA was isolated according to the method of Chomczynski and Sacchi [9]. RNA (10 µg/lane) was electrophoresed in a 1.0% or 1.2% agarose-formaldehyde gel and transferred to nylon or nitrocellulose membranes by capillary blotting. cDNA fragments for E-selectin [10], VCAM-1 [11], or β -actin were labeled with [^{32}P]dCTP by random priming. E-selectin, VCAM-1 and β -actin specific mRNA transcripts were detected by hybridization and autoradiography.

2.4. Nuclear protein extraction and gel mobility shift assay

Nuclear protein extracts were prepared as previously described [12]. The nuclear protein extract (20 µg) was incubated in a binding reaction with the ^{32}P -end-labeled duplex oligonucleotide probe for the NF- κB consensus sequence of the Ig promoter (5'-CAGAGGGGTCTTTC-CGAGAGGAAGCT-3') provided by K. Bomsztyk (University of Washington, Seattle, WA), and then electrophoresed in a 4% polyacrylamide gel as previously described, except that the binding buffer contained 0.05% NP-40 [13]. The gels were dried and directly autoradiographed using Kodak X-AR film.

2.5. Cytosolic protein kinase C

Protein kinase C activity was assayed in the cytosolic fraction after pretreatment with PMA. Subcellular extracts were prepared according to the methods of Ostrowski et al. [14]. Cytosolic PKC activity was assayed at 30°C in a final volume of 50 µl containing 50 µM [γ - ^{32}P]ATP, 10 mM MgCl_2 , 0.6 mM EDTA, 3.3 mM EGTA, 2 mM dithiothreitol, 20 mM HEPES, pH 7.5, and 0.7 mg/ml histone III-s or 0.25 mg/ml histone III-s *N*-bromosuccinimide fragment. PKC activity was assayed in the presence or absence of 2 mM CaCl_2 , 100 µM phosphatidylserine, and 60 µM diolein. After a 5 min incubation, 40 µl of the reaction mixture was spotted onto phosphocellulose filter papers and immersed in a 75 mM phosphoric acid wash. The papers were washed five times, blotted dry, and counted in a liquid scintillation counter.

2.6. Reagents

Tumor necrosis factor- α was a gift of R. Lobb (Biogen Inc., Cambridge, MA), interleukin- 1β was purchased from R&D Systems Inc. (Minneapolis, MN), lipopolysaccharide was purchased from List Biologicals Inc. (Campbell, CA) and [^{35}S]methionine was purchased from New England Nuclear (Wilmington, DE). All other reagents not previously detailed were purchased from standard commercial sources.

3. RESULTS

3.1. Induction of VCAM-1 surface protein expression by activators of PKC

Similar to its ability to induce E-selectin and ICAM-1 expression on HUVEC [2-4], the phorbol ester PMA induced VCAM-1 surface protein expression in a time- and concentration-dependent manner. Induction was

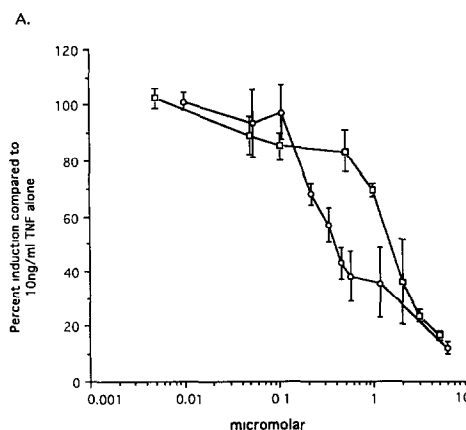


Fig. 2. Concentration-dependent inhibition of $\text{TNF-}\alpha$ -stimulated VCAM-1 surface expression by staurosporine and calphostin C. Calphostin C (\square) or staurosporine (\circ) were added to HUVEC in increasing concentrations, in dim light, 30 min prior to the addition of $\text{TNF-}\alpha$ (10 ng/ml). After an additional 5.5 h incubation at 37°C, surface protein expression of VCAM-1 was measured by ELISA. Data are expressed as percent induction in the presence of calphostin C or staurosporine compared to the induction with $\text{TNF-}\alpha$ alone (mean ± S.E.M., $n = 3$). The data are expressed as percent induction compared to $\text{TNF-}\alpha$ alone because the absolute OD₄₉₀ values are not directly comparable between assays. Induction of VCAM-1 surface expression by $\text{TNF-}\alpha$ (10 ng/ml) in 16 separate experiments resulted in a mean OD₄₉₀ value of 0.188 ± 0.023 .

Table I

Inhibition of the surface expression of E-selectin and VCAM-1 on HUVEC by staurosporine, calphostin C and Ro31-7549

	TNF (10 ng/ml)		Il-1 β (10 ng/ml)		LPS (100 ng/ml)		TCA precipitable counts (% of TNF control)
	E-selectin	VCAM-1	E-selectin	VCAM-1	E-selectin	VCAM-1	
Staurosporine (250 nM)	106 \pm 30% (n = 3)	51 \pm 14% (n = 3)	124 \pm 27% (n = 3)	22 \pm 16% (n = 3)	98 \pm 22% (n = 3)	23 \pm 13% (n = 3)	117 \pm 38% (n = 3)
Calphostin C (2 μ M)	104 \pm 12% (n = 3)	36 \pm 15% (n = 3)	115% (n = 1)	56% (n = 1)	102% (n = 1)	25% (n = 1)	106% (n = 1)
Ro31-7549 (10 μ M)	94 \pm 12% (n = 2)	49 \pm 5% (n = 2)					103 \pm 20% (n = 3)

Human umbilical vein endothelial cells were treated with staurosporine (10 nM to 1 μ M), calphostin C (50 nM to 4 μ M), or Ro31-7549 (1.25 μ M to 25 μ M) 30 min prior to the addition of TNF- α , Il-1 β , or LPS. After an additional 5.5 h incubation at 37°C, surface protein expression of E-selectin and VCAM-1 was measured by ELISA. Data are expressed as the percent induction in the presence of inhibitor compared to the induction with TNF- α , Il-1 β , or LPS alone. The concentrations of inhibitor listed were chosen because VCAM-1 surface expression was inhibited by approximately 50% or greater for all 3 agonists tested. Alternatively, HUVEC were pre-incubated with 20 μ Ci/ml [35 S]methionine for at least one hour before treatment of the plates. After a six hour incubation with TNF- α , Il-1 β , or LPS, total protein synthesis was measured after precipitation with trichloroacetic acid. At the concentrations tested, none of the inhibitors reduced protein synthesis compared to TNF- α alone.

maximal after a 6 h incubation at 37°C, and declined to baseline levels by 18 h despite the continuous presence of PMA (Fig. 1). Surface expression of VCAM-1 was observed at 50 ng/ml, and was maximal with 1 μ g/ml PMA. Phorbol dibutyrate (PDBU) gave similar results to PMA, while phorbol itself was without effect. The non-phorbol PKC activator mezerein also induced VCAM-1 surface protein expression, with maximal expression induced at 1 μ M. The extent of total phorbol ester-induced VCAM-1 and E-selectin surface expression is 2–6-fold less than the expression induced by optimal concentrations of TNF- α [15].

3.2. Effect of PKC inhibition or downregulation on surface expression of VCAM-1

To investigate the role of PKC in the signal transduction mechanisms by which Il-1 β , TNF- α , and LPS in-

duce the surface expression of VCAM-1 in HUVEC, we used staurosporine, calphostin C or Ro31-7549 to inhibit PKC [16–18], or pretreatment with PMA to downregulate PKC. Fig. 2 illustrates the concentration-dependent inhibition of TNF- α -induced surface expression of VCAM-1 by calphostin C or staurosporine. Similar results were obtained for inhibition of Il-1 β - and LPS-induced VCAM-1 surface expression (data not shown). In Table I, the concentrations of staurosporine, calphostin C and Ro31-7549 listed reduced TNF- α , Il-1 β -, or LPS-induced surface expression of VCAM-1 by 50% or greater, without significant effect on E-selectin surface protein expression, or on total TNF- α -induced protein synthesis. Expression of E-selectin surface protein was not affected until cytotoxic concentrations of the inhibitors were reached, indicating that the reduction in TNF- α -, Il-1 β -, or LPS-induced VCAM-1

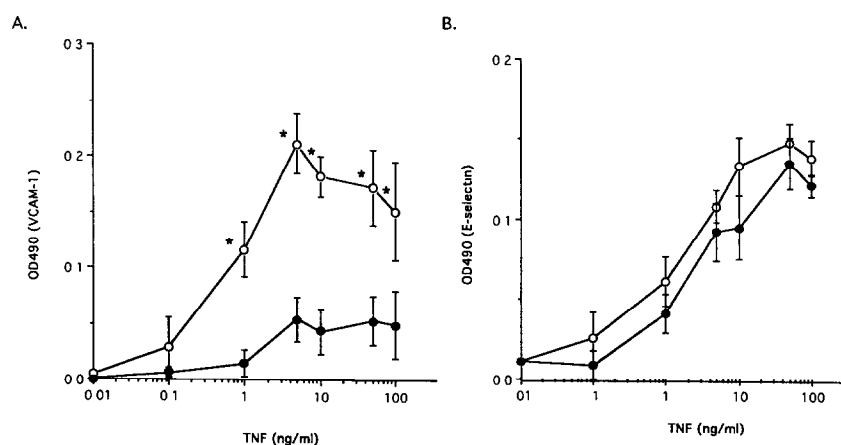


Fig. 3. Effect of pretreatment with PMA on induction of the surface expression of VCAM-1 and E-selectin by TNF- α . Human umbilical vein endothelial cells were treated for 24 h with PMA (100 ng/ml) and then TNF- α was added for an additional 5.5 h incubation at 37°C. Data are expressed as mean \pm S.E.M. (A) Induction of VCAM-1 by TNF- α in control (open circles) and PMA-pretreated (filled circles) HUVEC (means \pm S.E.M. of 5 experiments). (B) Induction of E-selectin by TNF- α in control (open circles) and PMA-pretreated (filled circles) HUVEC (means \pm S.E.M. of 5 experiments). * P < 0.05 compared to controls.

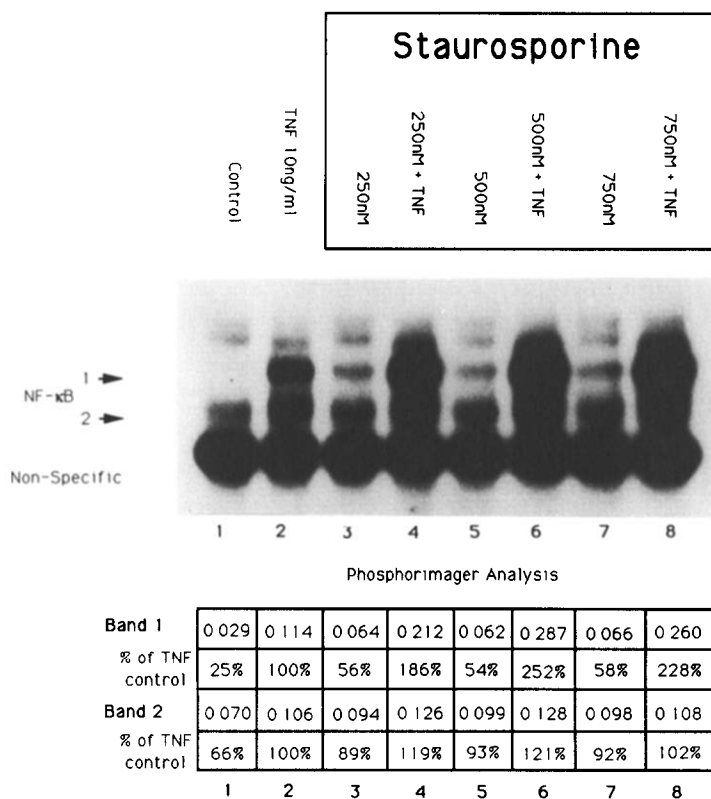


Fig. 4. Effect of staurosporine on TNF- α -stimulated NF- κ B-like binding activity. Human umbilical vein endothelial cells were stimulated with TNF- α (10 ng/ml) for 2 h, with or without pretreatment with staurosporine (250 nM to 750 nM) for 30 min. Nuclear proteins were extracted as described, incubated in a binding reaction with a radiolabeled NF- κ B oligonucleotide probe, and electrophoresed on a polyacrylamide gel. TNF- α -stimulated NF- κ B-like binding activity in the nuclear protein extract which specifically competed with a nonlabeled NF- κ B probe. Staurosporine did not prevent induction of this NF- κ B-like binding activity. The table lists the band densities for this individual experiment, measured by phosphorimaging and normalized to the total probe loaded per lane.

surface protein expression was not due to non-specific toxicity of the inhibitors.

Pretreatment with PMA (100 ng/ml; 160 nM) for 24 h was used to downregulate PKC [19]. Pretreatment of HUVEC with PMA (100 ng/ml) for 24 h resulted in an almost complete loss of cytosolic PKC activity using the *N*-bromosuccinimide fragment of histone III-s to measure PKC activity. Phosphorylation of the *N*-bromosuccinimide fragment of histone III-s was only 5.6 ± 3.3 cpm/ μ g protein for HUVEC pretreated with PMA compared to 95.3 ± 32.8 cpm/ μ g protein for control HUVEC ($n = 4$).

Pretreatment with PMA significantly reduced the ability of IL-1 β , TNF- α , and LPS to induce surface expression of VCAM-1. After a pretreatment with PMA, induction of VCAM-1 by TNF- α was only $22 \pm 12\%$, by IL-1 β $22 \pm 8\%$, and by LPS $33 \pm 5\%$ compared to control cells (Fig. 3A). In contrast, pretreatment with PMA did not reduce TNF- α -induced E-selectin surface expression (Fig. 3B). Pretreatment of HUVEC for 24 h with the PKC agonists phorbol dibutyrate or mezerein also inhibited TNF- α -induced VCAM-1, but not E-selectin surface protein expression (data not shown), illus-

trating the selective effect of a loss of PKC activity on VCAM-1 expression.

3.3. Effect of downregulation or inhibition of PKC on induction of NF- κ B-like binding activity

Interleukin-1 β , TNF- α , and LPS induced binding of nuclear protein extracts to a labeled NF- κ B oligonucleotide consensus sequence from the Ig promoter, which was specifically competed off by an unlabeled oligonucleotide containing the Ig NF- κ B binding sequence (data not shown). A prominent constitutive band was also present that did not compete for the unlabeled NF- κ B oligonucleotide probe, and was therefore designated nonspecific. The PKC inhibitor staurosporine, at concentrations that almost completely blocked TNF- α -induced VCAM-1 surface protein expression, did not reduce the ability of TNF- α to induce an NF- κ B-like binding activity, as depicted for one of four separate experiments in Fig. 4. By phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA) and following normalization to levels of total probe loaded, TNF- α -induced NF- κ B-like binding activity was $122 \pm 38\%$ in the presence of 250 nM staurosporine, $139 \pm 66\%$ in the

presence of 500 nM staurosporine, and $143 \pm 54\%$ (means \pm S.D.) in the presence of 750 nM staurosporine compared to 10 ng/ml TNF- α alone. Staurosporine itself induced a slight NF- κ B-like binding activity by a mechanism that is unclear at this time (Fig. 4, lanes 3, 5 and 7).

3.4. Effect of downregulation or inhibition of PKC on VCAM-1 mRNA accumulation

Interleukin-1 β , TNF- α , and LPS induced a significant increase in the accumulation of VCAM-1 mRNA within 4 h. No transcript was evident in HUVEC under normal, resting conditions (Fig. 5, lane 1). A 24 h pretreatment with PMA completely abolished the ability of a subsequent 4 h incubation with PMA to induce accumulation of VCAM-1 mRNA (Fig. 5, lane 10). Pretreatment with PMA also greatly diminished the accumulation of VCAM-1 mRNA in response to IL-1 β , TNF- α , and LPS (Fig. 5, lanes 7, 8, 9).

Similar to a pretreatment with PMA, the protein kinase C inhibitor staurosporine reduced the accumulation of VCAM-1 mRNA in response to TNF- α , at concentrations that also inhibited the surface expression of VCAM-1. At 250 nM, staurosporine diminished TNF- α -induced VCAM-1 mRNA accumulation by $45 \pm 1.3\%$, and at 500 nM, by $81 \pm 12\%$ ($n = 3$). At 750 nM, VCAM-1 mRNA accumulation was diminished by 88% ($n = 1$). In contrast to its effect on VCAM-1 mRNA accumulation, staurosporine did not diminish

E-selectin mRNA accumulation at 250 nM, and at 500 nM E-selectin mRNA accumulation was not significantly reduced ($81 \pm 17\%$ of TNF- α -induced mRNA accumulation, $n = 3$). Accumulation of E-selectin mRNA was not significantly reduced by staurosporine, consistent with its lack of inhibition of E-selectin surface protein expression. Regression analysis of the effect of staurosporine on VCAM-1 mRNA accumulation and surface protein expression revealed that inhibition of VCAM-1 mRNA accumulation and VCAM-1 surface protein expression were directly and linearly related with a correlation coefficient (r^2) of 0.965.

4. DISCUSSION

Our results demonstrate a role for protein kinase C in IL-1 β -, TNF- α -, and LPS-induced surface expression of VCAM-1 on HUVEC. Inhibitors of PKC or pretreatment with PMA for 24 h, which downregulates PKC, greatly diminished the extent of IL-1 β -, TNF- α -, and LPS-induced mRNA accumulation and surface expression of VCAM-1. An NF- κ B-like binding activity precedes the accumulation of E-selectin mRNA induced in HUVEC by IL-1 β , TNF- α , and LPS [13], and an NF- κ B-like factor has been shown to be essential, but not sufficient, for induction of E-selectin gene transcription [20]. The promoter region of the VCAM-1 gene also has consensus binding sequences for NF- κ B, and deletion or mutation of these NF- κ B binding sequences in the

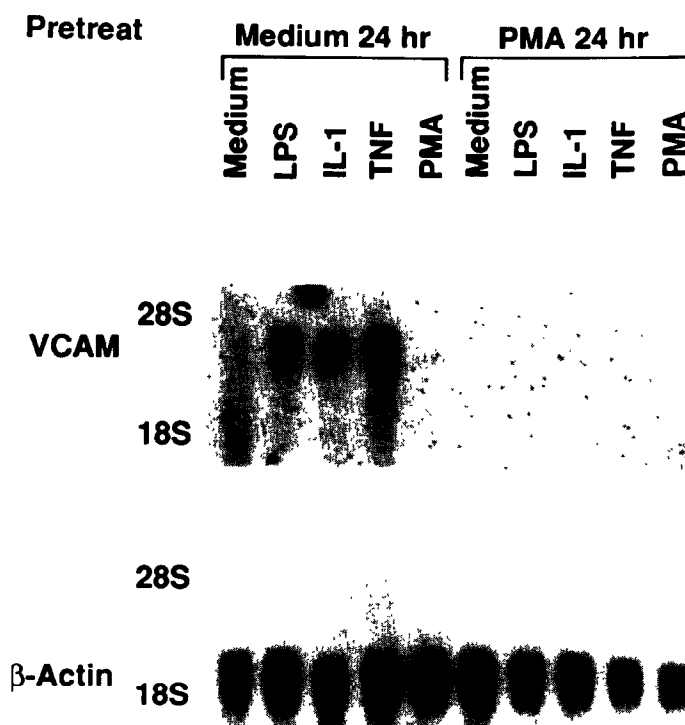


Fig. 5. Effect of pretreatment with PMA on IL-1 β -, TNF- α - and LPS-induced accumulation of VCAM-1 mRNA. Human umbilical vein endothelial cells were stimulated with IL-1 β , TNF- α , or LPS for 4 h, with or without a prior pretreatment with PMA (100 ng/ml) for 24 h. Total cellular RNA was extracted as described, and probed with 32 P-labeled cDNA for human VCAM-1 and β -actin.

VCAM-1 promoter prevents transcription [21,22]. However, neither staurosporine nor pretreatment with PMA prevented induction of this NF- κ B-like binding activity, indicating that induction of NF- κ B-like binding activity is not dependent upon PKC activation, nor is induction of NF- κ B-like binding activity sufficient for transcription of the VCAM-1 gene.

Acknowledgements: This work was supported by USPHS Grants POHL03174 and PO1HL30542.

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